Cell Reports, Volume *17*

Supplemental Information

Myeloid-Derived Suppressor Cells Are Controlled by Regulatory T Cells via TGF-β during Murine Colitis

Cho-Rong Lee, Yewon Kwak, Taewoo Yang, Jung Hyun Han, Sang-Heon Park, Michael B. Ye, Wongeun Lee, Kyu-Young Sim, Jung-Ah Kang, Yong-Chul Kim, Sarkis K. Mazmanian, and Sung-Gyoo Park

Supplemental Figures

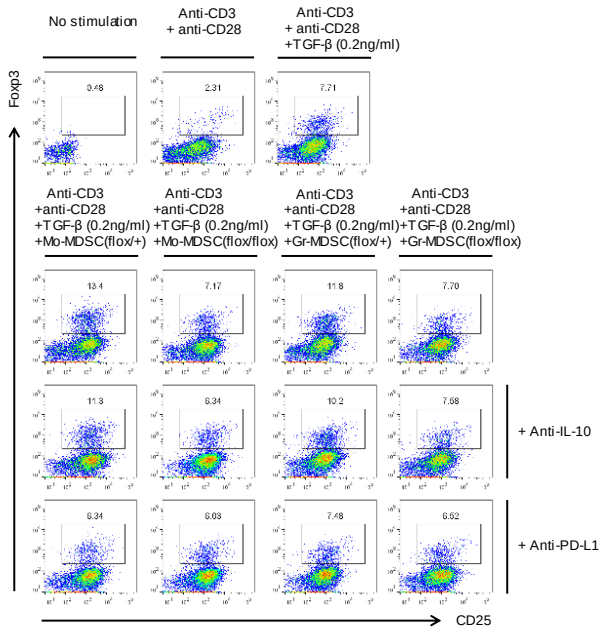
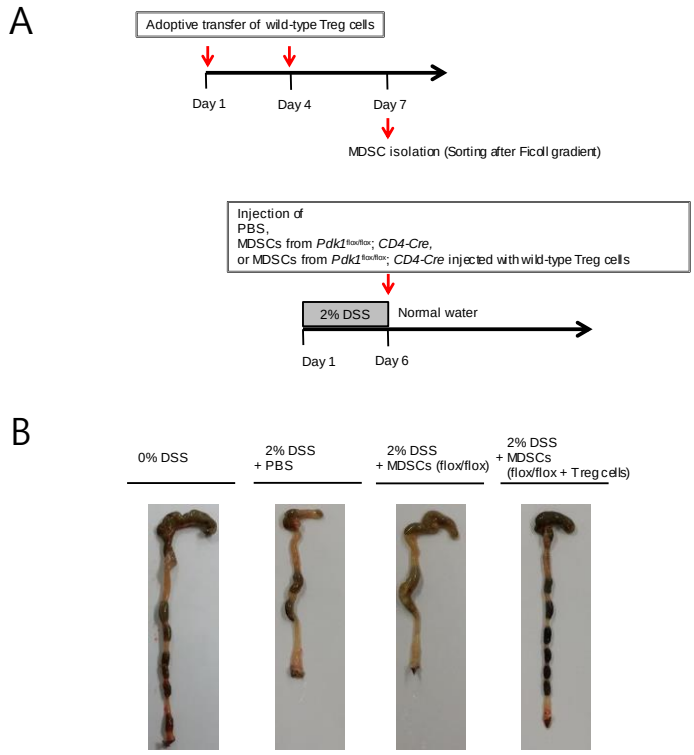


Figure S1 (Related to Figure 1). Flow cytometric analysis of *in vitro*-induced Treg cells with Mo-MDSCs and Gr-MDSCs isolated from the spleen. Data are representative of three independent experiments.



\

Figure S2 (Related to Figure 2). DSS–induced colitis for testing the function of MDSCs isolated from the spleen of *Pdk1*$^{flox/flox}$; *CD4-Cre* or *Pdk1*$^{flox/flox}$; *CD4-Cre* mice injected with wild-type Treg cells. (A) Experimental scheme. Inflammation of the intestines in mice was induced by DSS for testing the function of MDSCs isolated from the spleen of *Pdk1*$^{flox/flox}$; *CD4-Cre* or *Pdk1*$^{flox/flox}$; *CD4-Cre* mice injected with wild-type Treg cells. (B) Photomicrographs of mice colons. Inflammation of the intestines in mice was induced by DSS for testing the function of MDSCs isolated from the spleen of *Pdk1*$^{flox/flox}$; *CD4-Cre* or *Pdk1*$^{flox/flox}$; *CD4-Cre* mice injected with wild-type Treg cells.

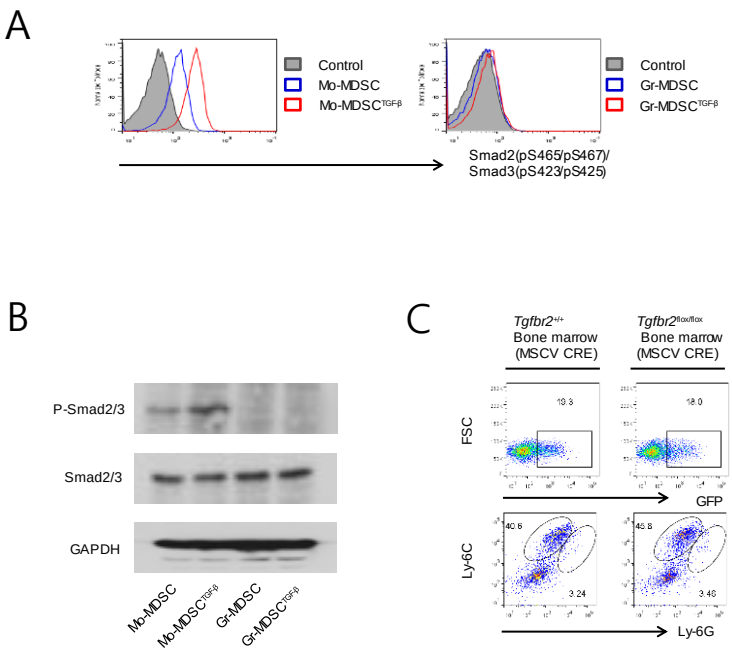


Figure S3 (Related to Figure 4). Analysis of TGF-β signaling in MDSC differentiation. (A) Flow cytometric analysis of phospho-Smad 2/3 levels in bone marrow-derived Mo-MDSCs or Gr-MDSCs differentiated with or without TGF-β. (B) Phospho-Smad2/3 levels in bone marrow-derived Mo-MDSCs or Gr-MDSCs differentiated with or without TGF-β were analyzed by immunoblot. (C) Flow cytometric analysis of subpopulation of MDSCs infected with recombinant retrovirus (MSCV-Cre-IRES–GFP). We isolated bone marrow cells from *Tgfbr2*$^{+/+}$ or *Tgfbr2*$^{flox/flox}$ mice, and subsequently incubated these cells with GM-CSF, IL-4 and 2-ME. After 24 hr, proliferating bone marrow cells were infected with Cre-expressing retrovirus and 2 days later the subpopulation of GFP^{+} bone marrow derived MDSCs were analyzed by flow cytometry.

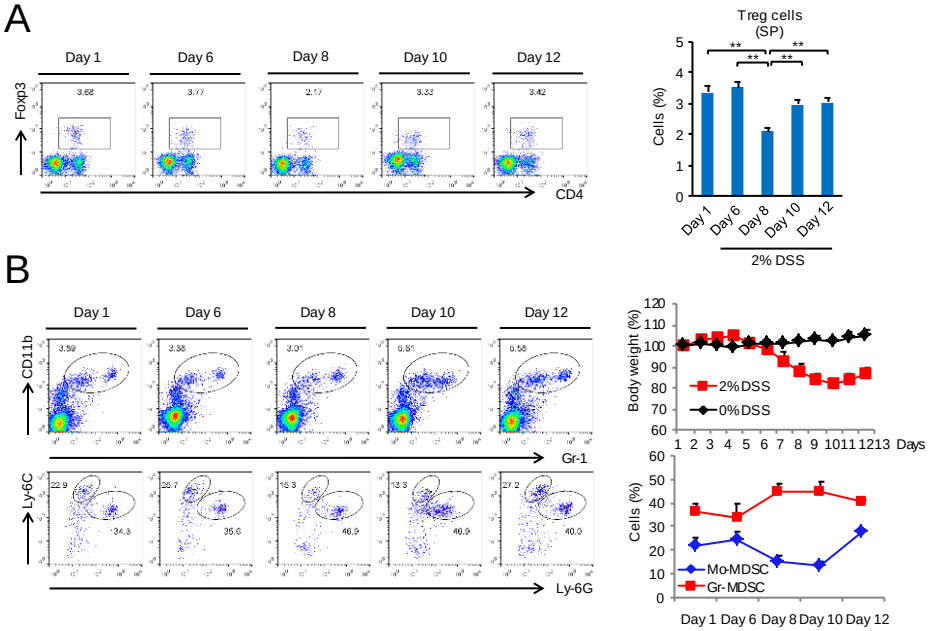


Figure S4 (Related to Figure 6). Analysis of Treg cell and MDSC populations in mice with DSS-induced colitis. (A) Flow cytometric analysis of Treg cells and the percentage of Treg cells among a population of cells isolated from the spleen of DSS-treated mice. (B) Flow cytometric analysis of MDSC subpopulations and the percentage of MDSC subpopulations among the total MDSCs isolated from the spleen of DSS-treated mice. Data are representative of two (A and B) independent experiments. Results are expressed as the mean ± SD. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA and Tukey post-test (A).

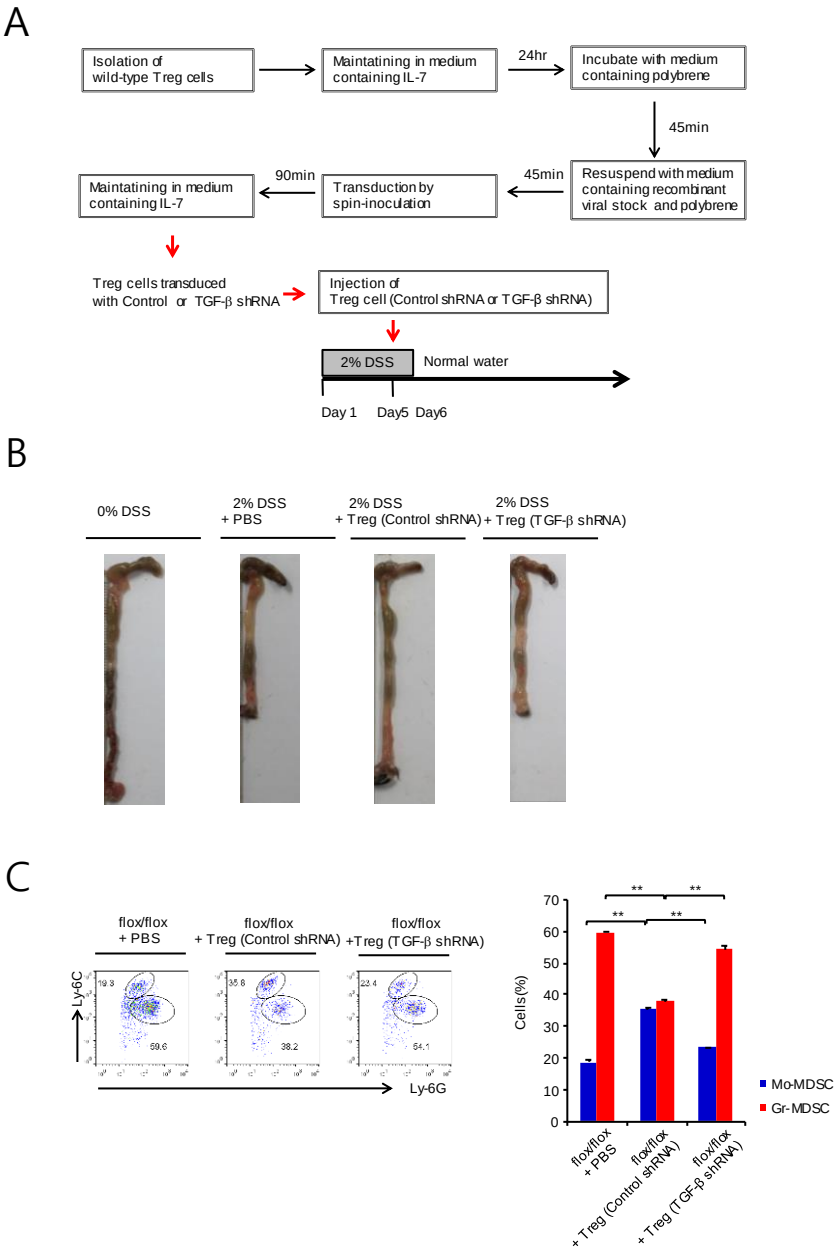


Figure S5 (Related to Figure 6). Analysis of MDSCs regulated by Treg cell-derived TGF-β. (A) Experimental scheme. Inflammation of the intestines in mice was induced by DSS to test the effects, in Treg cells, of TGF-β shRNA knock-down (or a control shRNA) on MDSC differentiation. (B) Photomicrographs of mice colons. Inflammation of the intestines in mice was induced by DSS to test the effects, in Treg cells, of TGF-β shRNA knock-down (or a control shRNA) on MDSC differentiation. (C) Regulation of MDSC subpopulation differentiation by Treg cell-derived TGF-β. The percentage of MDSC subpopulations from the spleen of *Pdk-1*$^{flox/flox}$;*CD4-Cre* (flox/flox) mice (each group, n = 3) after adoptive transfer of control shRNA-transduced or TGF-β shRNA-transduced Treg cells (4×10^5 cells). Results are expressed as the mean ± SD. *, $p < 0.05$; **, $p < 0.01$ by two-way ANOVA and bonferroni post-test (C).

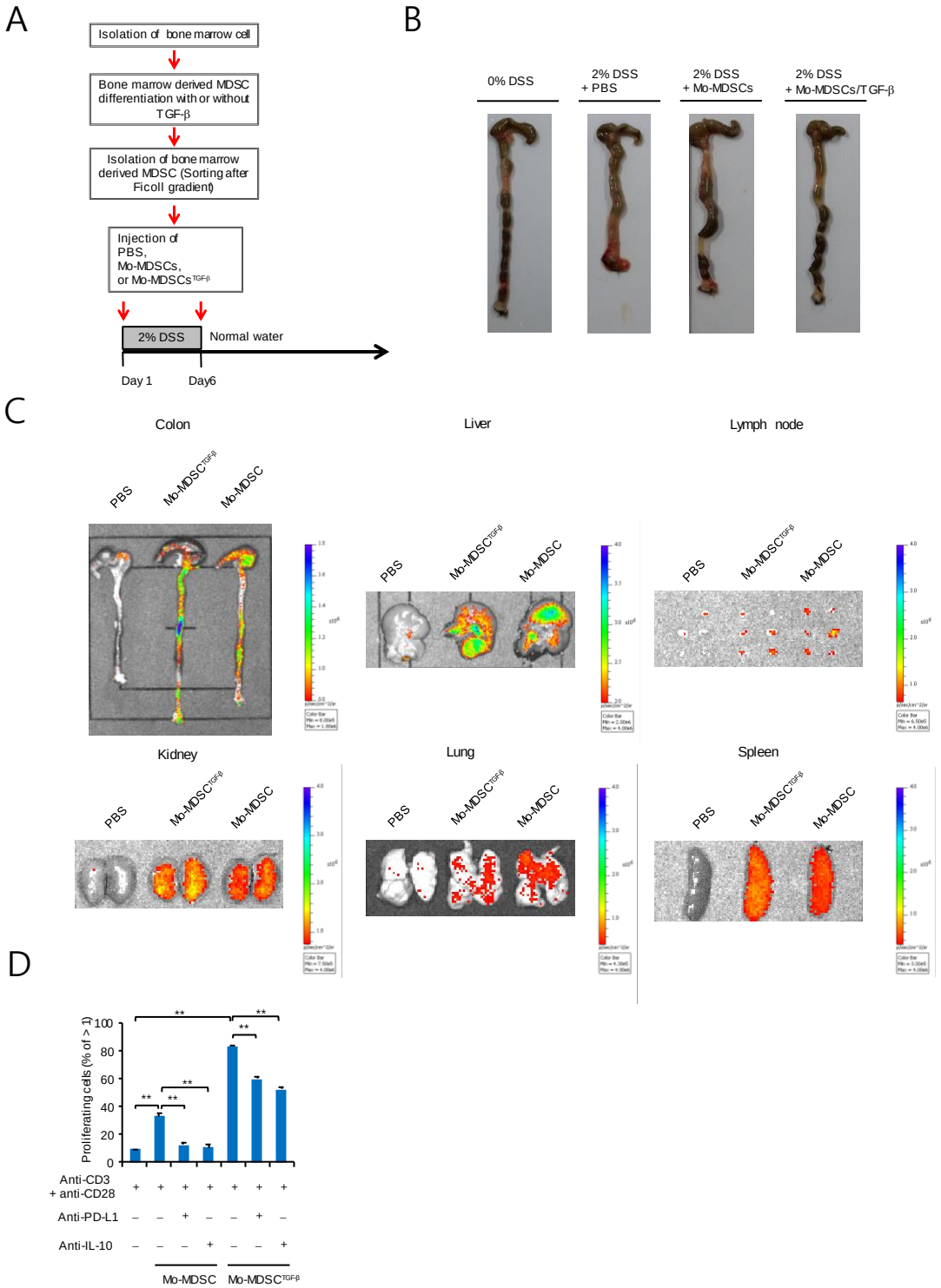


Figure S6 (Related to Figure 7). Analysis of immune suppressive function of bone marrow derived Mo-MDSCs differentiated with or without TGF-β. (A) Experimental scheme. Inflammation of the intestines in mice was induced by DSS for testing the function of bone marrow-derived Mo-MDSC or bone marrow-derived Mo-MDSC$^{TGF-β}$. (B) Photomicrographs of mice colons. Inflammation of the intestines in mice was induced by DSS for testing the function of bone marrow-derived Mo-MDSC or bone marrow-derived Mo-MDSC$^{TGF-β}$. (C) Fluorescence imaging analysis. Mice were treated with DSS for 6 days and injected with DiI-labeled bone marrow-derived Mo-MDSC or Mo-MDSC$^{TGF-β}$ (4×10^6 cells). After 36 hr, fluorescence images of each organ were obtained using the IVIS 200 imaging system. (D) Treg cell expansion analysis. Flow cytometric analysis of proliferating CFSE-labeled Treg cells in the presence of bone marrow-derived Mo-MDSC or Mo-MDSC$^{TGF-β}$ with or without anti-PD-L1 antibody and anti-IL-10 antibody. Data are representative of two independent experiments. Results are expressed as the mean ± SD. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA and Tukey post-test (D).

Supplemental Experimental Procedures

In vitro Treg cell differentiation

Treg cells were generated *in vitro* from $CD4^+CD25^-$ T-cells from C57BL/6 mice by stimulation with plate-bound anti-CD3 (17A2, Bio X Cell) and anti-CD28 (145-2C11, Bio X cell) for 54 hr in the presence of TGF-β (0.2 ng/ml)(eBioscience), which is defined as a suboptimal condition for differentiating Treg cells *in vitro*. For blocking of IL-10 and PD-L1, we used anti-IL-10 (10 μg/ml) (JES5-2A5, eBioscience) and anti-PD-L1 antibodies (10 μg/ml) (MIH5, eBioscience).

ARG1 enzymatic activity assay

ARG1 activity was measured in cell lysates prepared by lysing cells for 30 min with 100 μl of 0.1% Triton X-100. Next, 100 μl of 25 mM Tris-HCl and 10 μl of 10 mM $MnCl_2$ were added, and the enzyme was activated by heating for 10 min at 56°C. Arginine hydrolysis was performed by incubating the lysate with 100 μl of 0.5 M L-arginine (pH 9.7) at 37°C for 2 hr. The reaction was stopped by adding 900 μl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). The concentration of urea was measured at 540 nm after the addition of 40 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol), followed by heating at 95°C for 30 min.

Histology and scoring.

The stained intestinal sections were graded by a blinded scorer. Histological grading was based on observed inflammation (0–4) and observed epithelial injury (0–4). Clinical scores of observed inflammation were defined as follows: 0, no inflammation; 1, low level of inflammation with mildly increased inflammatory cells in the lamina propria; 2, moderately increased inflammation in the lamina propria (multiple foci); 3, a high level of inflammation with evidence of wall thickening by inflammation; and 4, severe inflammation with transmural leukocyte infiltration and/or architectural distortion. Grading scores of observed epithelial injury was defined as follows: 0, normal or no infiltration by neutrophils; 1, occasional epithelial lesions (focal and superficial or rare cryptitis); 2, foci of cryptitis, including rare crypt abscess; 3, multiple crypt abscess and/or focal ulceration; and 4, extensive ulceration.

TGF-β knockdown in Treg cells.

Treg cells were cultured in RPMI 1640 medium (HyClone) supplemented with 5% FBS (HyClone) and 20 ng/ml IL-7 (eBioscience). The cultures were maintained in 6-well plates at 37°C in a 5% CO_2-humidified environment. After 24 hr, cells were pelleted by centrifugation and resuspended with pre-warmed medium containing polybrene (6 μg/ml) (Sigma-Aldrich) and incubated for 45 min in a 37 °C incubator. Next, the cells were resuspended with pre-warmed recombinant viral stock; control shRNA lentiviral particles or TGF-β lentiviral particles (Santa Cruz Biotechnology, Inc.) containing polybrene 6 μg/ml in 24-well plates. After incubation for 45 min at 37°C incubator, the plates were centrifuged at 2,250 rpm for 90 min at 37°C. Finally, the medium containing virus was removed and fresh medium containing IL-7 added for 1 hr in a 37°C incubator. Cells were washed with PBS and injected intravenously into DSS-treated mice.

***In vitro* suppression assays.**

$CD4^+$ T cells (1.5 x 10^5 cells/well) were isolated from the spleen or lymph nodes by negative isolation and cultured in 96-well plates with Dynabeads (Invitrogen) coated with anti-CD3 and anti-CD28 antibodies. MDSCs were isolated via FACSAria III flow cytometer (BD Biosciences) on the basis of cell surface marker staining and isolated cells (3 x 10^5 cells/well) were co-cultured with $CD4^+$ T cells. After 54 hr of co-culture, ^3H-thymidine (1 μCi/well) was added to each well, and the cells were labeled for an additional 18 hr. Cells were harvested on glass filters and radioactivity was measured using a liquid scintillation counter (Beckman Coulter, Inc).

Fluorescence imaging.

MDSCs were isolated via FACS Aria III flow cytometer (BD Biosciences) on the basis of cell surface marker staining. The isolated subpopulation of cells were stained in suspension (4 x 10^6 cells in 0.5 ml PBS) with 5 μM of DiI Stain (D-282, Invitrogen) for 15 min in a 37°C incubator. Cells were then washed twice with PBS and injected intravenously into DSS-treated mice. Mice were sacrificed and their organs were excised and imaged 36 hr after DiI labeled cells were administered intravenously. DiI fluorescence was measured *in vivo* using the IVIS 200 imaging system (Caliper). The fluorescence of each tissue sample was obtained by subtracting the fluorescence intensity of each corresponding tissue from a blank (non-fluorescent) mouse. The excitation and emission filter set in the IVIS was 535 nm and 600 nm, respectively, and the wavelength closest to the peak emission/excitation of the dye was selected among available IVIS filters.